E.2 His-tagging of mmo-genes

All six MMO genes, already cloned in the shipping vector, are amplified with His-Tag Primers. Afterward the tagged genes are cloned into expression vectors containing an inducible Lac Promoter for further testing of protein expression.

07.05.2014

HIS-Tag attachment via PCR with synthezised primers

Sample	Gene	Plasmid		Primer		H_2O
		Nr.	Length [bp]	fw	rev	
1	mmoB	7	450	51	90	16,3 µL
2	mmoC (mut)	53	1050	53	91	14,1 µl
3	mmoD	29	362	55	92	13,1 µl
4	mmoX (2x mut)	49	1600	57	93	16,7 µl
5	mmoY (mut)	45	1250	59	94	17,0 µl
6	mmoZ	17	563	61	95	14,3 µl

Per PCR-sample:

- 25 µl Q5 High-Fidelity 2x Mastermix
- 2,5 µl Primer 1
- 2,5 µl Primer 2
- Template-DNA
    - 3,7 µl mmoB
    - 5,9 µl mmoC
    - 6,9 µl mmoD
    - 3,3 µl mmoX
    - 3,0 µl mmoY
    - 5,7 µl mmoZ
- Fill with H2O to 50 µl

Restriction of PCR- fragments and vector IGEM2014-6.3.11

100 µl PCR-Fragment
12 µl CutSmart Buffer 10x
1 µl Xba
1 µl PstI
6 µl H2O

25 µl IGEM2014-6.3.11
3 µl CutSmart Buffer 10x
1 µl Spe
1 µl PstI

Incubate at 37 °C for about 1h

08.05.2014

Purification of Fragments using Wizard SV Gel Kit (Promega)

Elution in 40 µl H2O

Ligation of cut PCR-Fragments and vector IGEM2014-6.3.11

2 µl vector

7 µl PCR-fragment

1 µl Buffer T4

0,5 µl T4 Ligase

- Incubate for 30 min at RT

Transformation

See protocol of transformation of E.coli using heat shock

09.05.2014

Colony-PCR

Construct	Clones
mmoB-HIS	1-8
mmoC-HIS	9-16
mmoD-HIS	17-24
mmoX-HIS	33-40
mmoY (45) -HIS	25-32
mmoY(46) -HIS	49-56
mmoY(47) -HIS	57-64
mmoY(48) -HIS	65-72
mmoZ-HIS	41-48

	Comment	Amount per Sample [µL]
Template	Colony	0,25
5x GoTaq-Buffer	No Mg2+	2
10 mM dNTPs		0,2
Primer 86	10 pmol per µL	0,5
Primer 87	10 pmol per µL	0,5
GoTaq Polymerase	5 U per µL (contains Loading Buffer)	0,05
MgCl		0,8
dH2O		5,7
Sum		10

	Temperature	Time [min]
1	95 °C	2:00
2	94 °C	0:15
3	56 °C	0:20
4	72 °C	1:10
5	Goto step 2 24x	
6	72 °C	3:00
7	16 °C	For ever

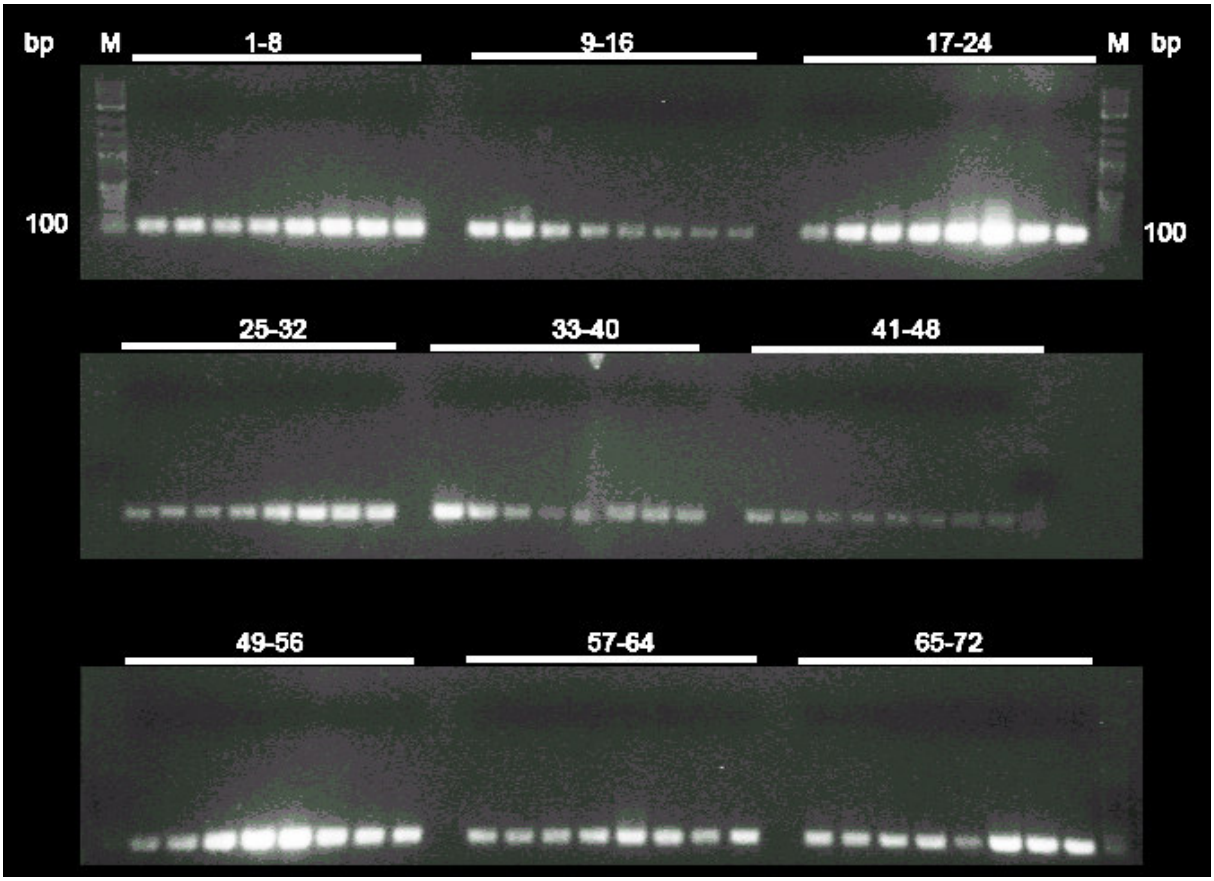


Fig. 2.1: *Colony-PCR - 1-8: mmoB-HIS; 9-16: mmoC-HIS; 17-24: mmoD-HIS; 25-32: mmoY-HIS (GlyStock 45); 33-40: mmoX-HIS; 41-48: mmoZ-HIS; 49-56: mmoY-HIS (GlyStock 46); 57-64: mmoY-HIS (GlyStock 47); 65-72: mmoY-HIS (GlyStock 48)*

11.05.2014

Colony-PCR (repeat) -> using new colonies than in the colony-pcr before

Construct	Clones
mmoB-HIS	1-8
mmoC-HIS	9-16
mmoD-HIS	17-24
mmoX-HIS	33-40
mmoY (45) -HIS	25-32
mmoY(46) -HIS	49-56

mmoY(47) -HIS	57-64
mmoY(48) -HIS	65-72
mmoZ-HIS	41-48

	Comment	Amount per Sample [µL]
Template	Colony	-
5x GoTaq-Buffer	No Mg2+	2
10 mM dNTPs		0,2
Primer 86	10 pmol per µL	0,5
Primer 87	10 pmol per µL	0,5
GoTaq Polymerase	5 U per µL (contains Loading Buffer)	0,05
MgCl		0,8
dH2O		5,95
Sum		10

	Temperature	**Time [min]**
1	95 °C	2:00
2	94 °C	0:15
3	56 °C	0:20
4	72 °C	1:10
5	Goto step 2 24x	
6	72 °C	3:00
7	16 °C	For ever

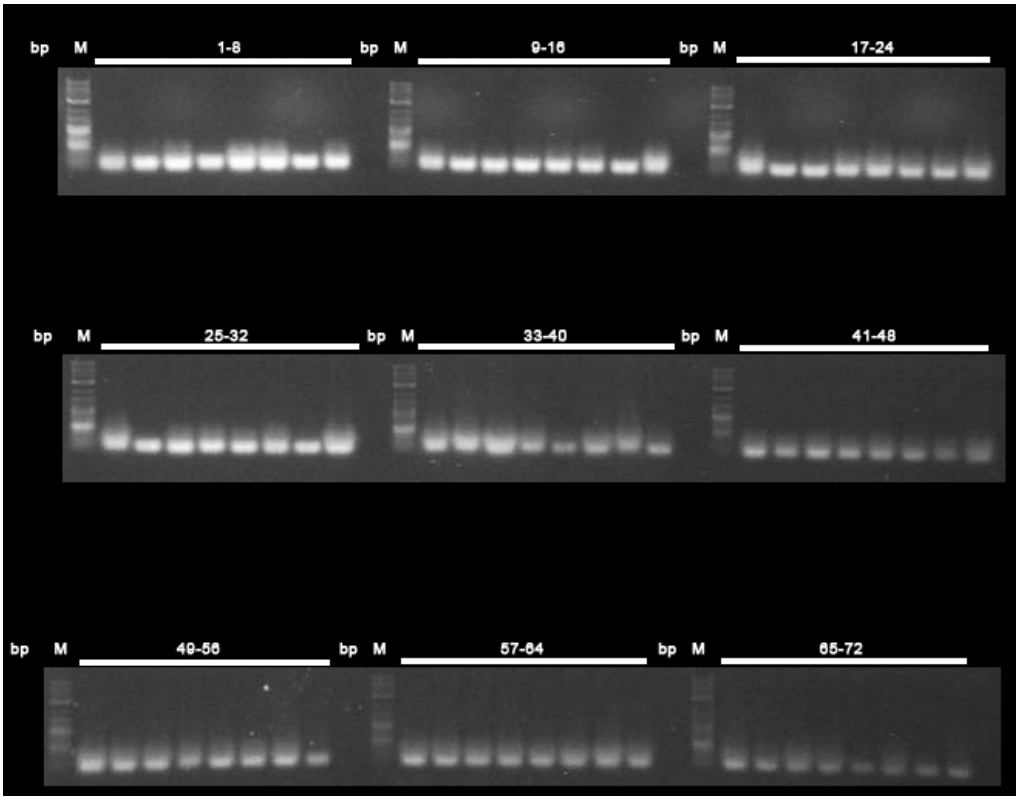


Fig. 2.2: *Colony-PCR - 1-8: mmoB-HIS; 9-16: mmoC-HIS; 17-24: mmoD-HIS; 25-32: mmoY-HIS (GlyStock 45); 33-40: mmoX-HIS; 41-48: mmoZ-HIS; 49-56: mmoY-HIS (GlyStock 46); 57-64: mmoY-HIS (GlyStock 47); 65-72: mmoY-HIS (GlyStock 48)*

12.05.2014

Restriction of vector IGEM2014-6.3.15

iGEM2014-6.3.15	25 µL
Cut-Smart Buffer	3 µL
SpeI	1 µL
PstI	1 µL
H_2O	0 µL
∑	30 µL

Incubate at 37 °C for about 1h

Purification of Fragments using Wizard SV Gel Kit (Promega)

Elution in 40 µl H2O

Ligation of cut PCR-Fragments (07.05.2014) and vector iGEM2014-6.3.15

2 µl vector

7 µl PCR-fragment

1 µl Buffer T4

0,5 µl T4 Ligase

- Incubate for 1h at RT

Inactivation: 20 min, 70°C

Transformation

See protocol of transformation of E.coli using heat shock

13.05.2014

Colony-PCR see 9.05.2014

mmoB 1-8	mmoX 25-31
mmoC 9-16	mmoY 33-39
mmoD 17-23	mmoZ 41-47

Test gel 1,0% (sample 5 and 8 were changed)

Repeat restriction (see 7.05.2014)

With alterations: -vector (iGEM2014-2.1 B0015 TT) cut with EcoRI and XbaI + 1 µL CIP (alkaline phosphates)

-Insert cut with SpeI and EcoRI

-gelectrophoresis of the restricted fragments in an 1,5% gel

-purification of fragments from gel

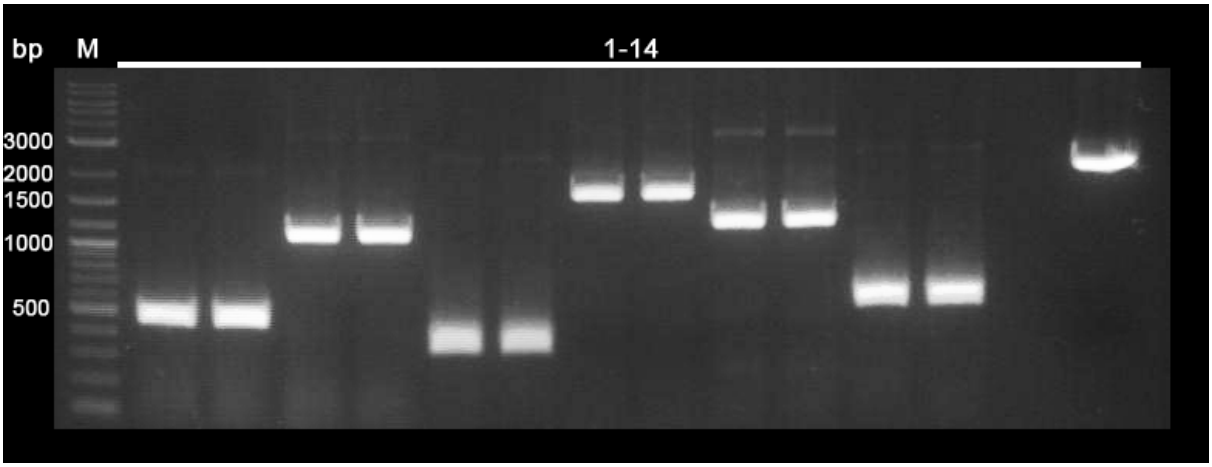


Fig. 2.3: *Gel purification - 1-2: mmoB; 3-4: mmoC (mutated); 5-6: mmoD; 7-8: mmoX (double-mutated), 9-10: mmoY (mutated); 11-12: mmoZ*

gel purification repeat for His-constructs and vector

14.05.2014

Test gel for the restriction sides

Vector 2.1	106 µL
Cut-Smart Buffer	10 µL
EcoRI	2 µL
XbaI	2 µL

Vector 2.1	8,5 µL
Cut-Samart Buffer	1,0 µL
EcoRI	0,5 µL

Vector 2.1	8,5 µL
Cut-Smart Buffer	1,0 µL
EcoRI	0,5 µL

Vector 2.1	8,5 µL

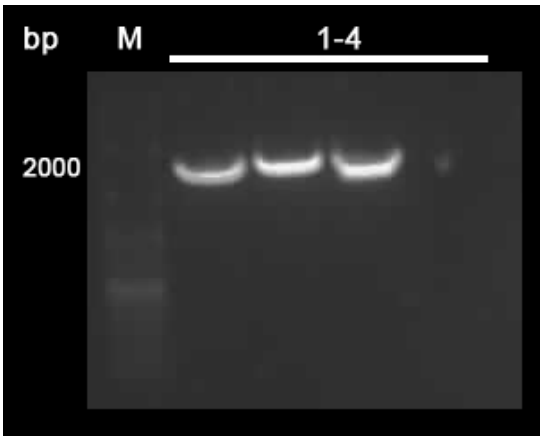


Fig. 2.4 *Test restriction - 1: Vector 2.1 (EcoRI+XbaI cut); 2: Vector2.1 (EcoRI cut); 3: Vector2.1 (XbaI); 4: Vector 2.1 (uncut)*

Test of the restriction sides (1:vector double cutted; 2: vector EcoRI; 3:vector XbaI; 4: vector not cut)

→Band of sample 4 could not be observed because the sample contained neither Glycerin nor loading Buffer

Carsten, Christian, Rüdiger

19.05.2014

<u>Testrestriction of B0015 TT vector:</u>

Restriction

	uncut	Cut with XbaI
Plasmid	5 µL	5 µL
Puffer	/	1 µL
XbaI	/	0,5 µL
H_2O	5 µL	3,5 µL

gelelectrophoresis

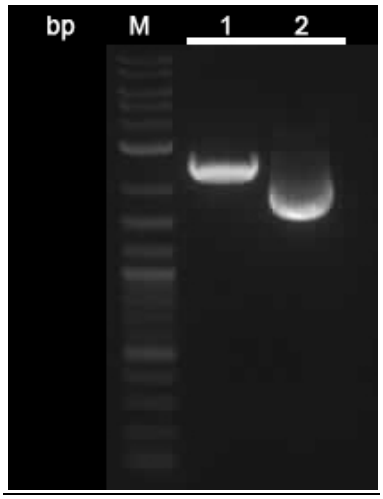


Fig. 2.5 *Test restriction - 1: B0015[TT] (XbaI cut); 2: B0015[TT] (uncut)*

(1: vector cut with XbaI; 2:vector uncut)

→Experiment showed that XbaI restriction site is active in vector B0015.

PCR amplification of mmo fragments and new TT-Plasmid

Sample	**Gene**	**Plasmid**		**Primer**		**H_2O**
		Nr.	**Length [bp]**	**fw**	**rev**	
1	mmoB	7	450	51	90	16,3 µL
2	mmoC	53	1050	53	91	14,1 µl
3	mmoD	29	362	55	92	13,1 µl
4	mmoX	49	1600	57	93	16,7 µl
5	mmoY	45	1250	59	94	17,0 µl
6	mmoZ	17	563	61	95	14,3 µl
7	B0015 (new)			88	89	

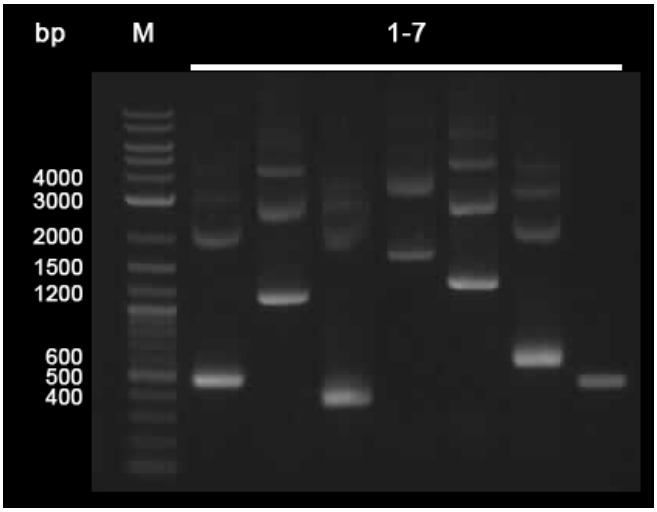


Fig. 2.6 PCR amplification – 1: mmoB; 2: mmoC; 3: mmoD; 4: mmoX; 5: mmoY; 6: mmoZ; 7: B0015[TT]

No pre-diluted plasmid was used for PCR, which lead to additional bands (vectors).

20.05.2014

Gel-purification of PCR-samples

Purification in 1% agarose gel. Designated bands are cut out and purificated with Promega Wizard PCR and Gel Purification Kit.

Christian, Lukas, Rüdiger

21.05.2014

Colony PCR of transformed overnight culture

-90 colonies were picked

-used primers 88/89

	Comment	**Volume per sample [µL]**	**Volume for X samples [µL]**
Template	Colony	0,25	27,5
5 x GoTaq-Puffer	contains NO Mg^{2+}	2	220
10 mM dNTPs		0,2	22
Primer 88	10 pmol μL^{-1}	0,5	55
Primer 89	10 pmol μL^{-1}	0,5	55
GoTaq Polymerase	5 U μL^{-1} (contains loading buffer)	0,05	5,5
MgCl		0,8	88
dH_2O		5,7	627
∑		10	1100

Gelelectrophoresis

-in 1% agarose gel at 160 V

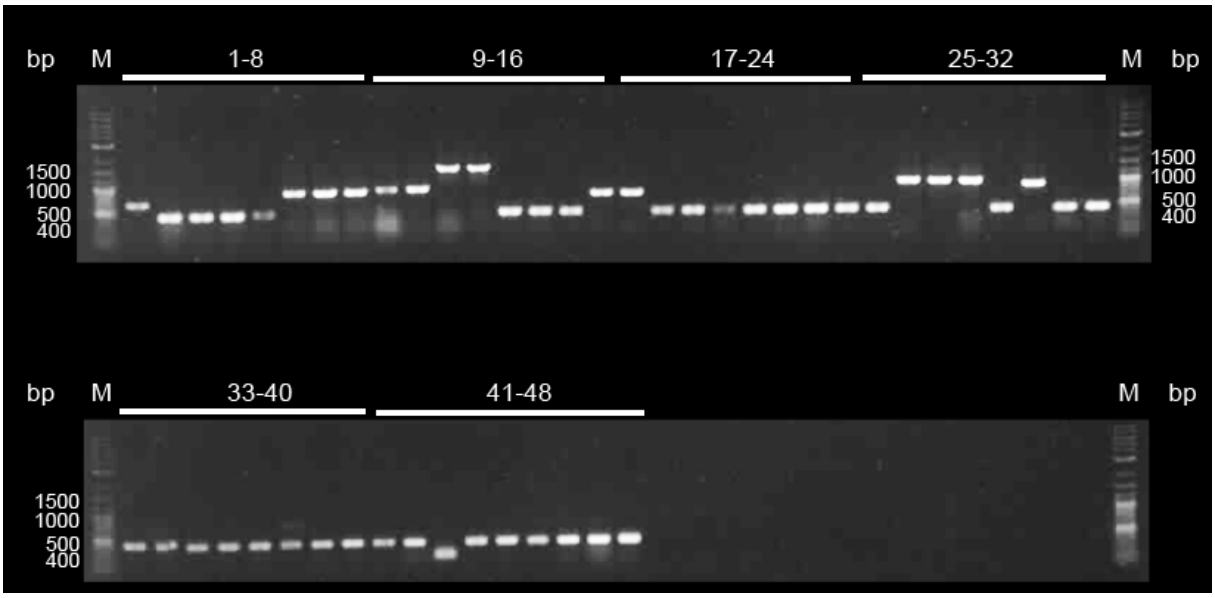


Fig. 2.7 *Colony-PCR - 1: mmoD ; 2-3: mmoX ; 4: mmoY ; 5-8: mmoZ ; 9-10: mmoB+B0015[TT] ; 11-14: mmoC+B0015[TT]; 15-17: mmoD+B0015[TT]; 18-20: mmoX+B0015[TT]; 21-25: mmoY+B0015[TT]; 26-28: mmoZ+B0015[TT]; 29-31: mmoB+B0015[TT] ; 32-35: mmoC+B0015[TT] ; 36-38: mmoD+B0015[TT]; 39-43: mmoX+B0015[TT]; 44-46: mmoY+B0015[TT]; 47-49: mmoZ+B0015[TT]*

Rüdiger, Lukas, Christian

22.05.2014

Colony PCR of mmoX

46 samples of mmoX

	Comment	**Volume per sample [µL]**	**Volume for 46 samples [µL]**
Template	Colony	0,25	27,5
5 x GoTaq-Puffer	contains No Mg^{2+}	2	165
10 mM dNTPs		0,2	16,5
Primer 1	10 pmol μL^{-1}	0,5	41,25
Primer 2	10 pmol μL^{-1}	0,5	41,25
GoTaq Polymerase	5 U μL^{-1} (contains loading buffer)	0,05	4,125
MgCl		0,8	66
dH_2O		5,7	470,25
Σ		10	804,375

Sample 11,12,13,16,17,18 failed, probably because they dried out in the thermocycler

27.05.2014

Restriction of Lac-Promoter and fragments

- Cutting of Lac-Promotor with SpeI and PstI
- Cutting of gene-fragments with XbaI and PstI

Ligation of Lac-Promoter and fragments

Transformation

- No colony-growth

28.05.2014

Mini PlasmidPrep of the fragments B,C,D(1/2) from the overnight culture

Plasmid	Concentration
mmoB + HIS + TT	206,82
mmoC + HIS + TT	185,08
mmoY + HIS + TT	220,68
mmoD 1 + HIS + TT	106,88
mmoD 2 + HIS + TT	139,09

Repeat-ligation of the restricted fragments/vector from 27.05.2014

- The restriction-samples of the last day are ligated again

New-amplification of Gene-HIS-constructs (fragments)

- PCR-amplification of the gene-HIS-constructs to have new plasmids for later use
- Purification of amplified fragments in 1% agarose gel

Transformation of the Ligation (repeated)

New restriction of the Fragments (elution from 27.5.)

01.06.2014

XL1-overnight culture

For competent cells

Glycerin stock revive of Gene-fragments and Lac-promoter

Glycerin stocks: 7,16,33,44,48,53,55

02.06.2014

Midi prep of revived glycerin stocks (fragments + lac promoter)

Construct	Concentration [ng/µl]
iGEM2014-4.-3.6.1 mmoB	524,77
iGEM2014-4.-3.10.5 mmoY	862
iGEM2014-6.3.11 LacProm [R0011] + RBS	354,69
iGEM2014-7.4.3.10.1-1 mmoY	696,18
iGEM2014-7.4.3.9.13-1/1 mmoX 2. Mutation	954,78
iGEM2014-7.4.3.7.10/2 mmoC	252,96

iGEM2014-9.1/1 mmoD (EcoRI-korrigiert) Masterplatten-Nr. 49	360,92

03.06.2014

Midi prep of revived glycerin stocks (fragments)

Construct	Concentration [ng/µl]
Mmoz	564,2

PCR

Template		Mastermix	Primer		H_2O
Gene	**volume**		**forward**	**reverse**	
mmoB+His+TT (77)	5ng	Q5 High fidelity 2x 12,5µl	88 1,25 µL	89 1,25 µL	Ad 25 µL
mmoC+His+TT (78)	5ng	Q5 High fidelity 2x 12,5µl	88 1,25 µL	89 1,25 µL	Ad 25 µL
mmoD+His+TT (80)	5ng	Q5 High fidelity 2x 12,5µl	88 1,25 µL	89 1,25 µL	Ad 25 µL
mmoX+His+TT (75)	5ng	Q5 High fidelity 2x 12,5µl	88 1,25 µL	89 1,25 µL	Ad 25 µL
mmoY+His+TT (79)	5ng	Q5 High fidelity 2x 12,5µl	88 1,25 µL	89 1,25 µL	Ad 25 µL
mmoZ+His+TT (66)	5ng	Q5 High fidelity 2x 12,5µl	88 1,25 µL	89 1,25 µL	Ad 25 µL

Two Samples per fragment

Restriction of HIS-fragments

- Cutting of Lac-Promotor with SpeI and PstI
- Cutting of gene-fragments with XbaI and PstI

Gelelectrophoresis and Gelelution

gelelectrophoresis in 0,8% (mmo C,X,Y) and 1,5% (mmo B,D,Z) agarose gel at 100 V

0,8 % Gel

Fragment	mmoC	mmoC	mmoX	mmoX	mmoY	mmoY
Expected length [bp]	1047	1047	1584	1584	1170	1170
Band-No.	1	2	3	4	5	6

1,5% Gel

Fragment	mmoB	mmoB	mmoD	mmoD	mmo Z	mmoZ
Expected length [bp]	426	426	312	312	513	513
Band-No.	1	2	3	4	5	6

Ligation of cut fragments (3.6.14) and vector

Transformation

4.6.2014

Colony-PCR of final fragments

Construct	Clones
mmoB-HIS	1-8
mmoC-HIS	9-16
mmoD-HIS	17-24
mmoX-HIS	25-32
mmoY -HIS	33-40
mmoZ-HIS	41-48

Primer 86/87

Fragment HIS	PCR sample	Master plate (position)
Mmoy	34	35
Mmoy	35	36
Mmoy	36	34

06.06.2014

Midi-Prep of His-fragments (B-Z).

11.06.2014

Colony PCR of transformed fragments

-Primer 88/89

	Comment	**Volume per sample [µL]**	**Volume for 100 samples [µL]**
Template	Colony	0,25	25

5 x GoTaq-Puffer	contains NO Mg^{2+}	2	200
10 mM dNTPs		0,2	20
Primer 1	10 pmol μL^{-1}	0,5	50
Primer 2	10 pmol μL^{-1}	0,5	50
GoTaq Polymerase	5 U μL^{-1} (contains loading buffer)	0,05	5
MgCl		0,8	80
dH_2O		5,7	570
∑		10	1000

Gelelectrophoresis in 1% agaorese gel at 140V

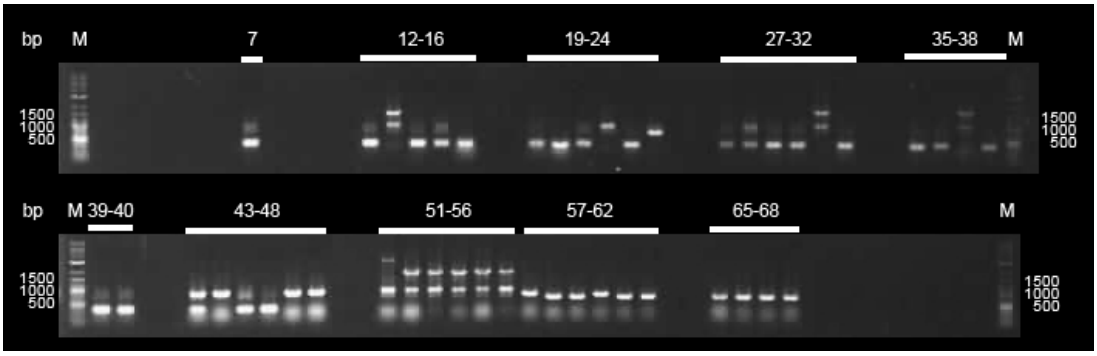
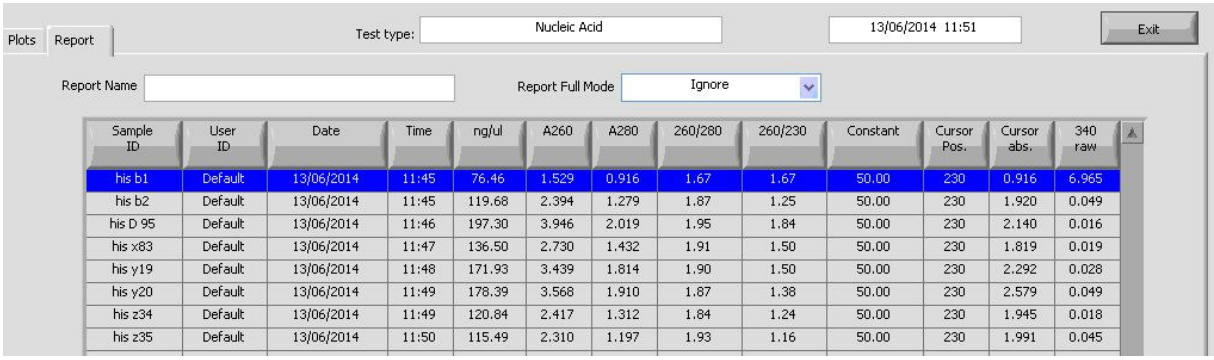


Fig. 2.8 Colony-PCRof His-fragments

13.06.2014

Mini-Prep of successfully cloned His-fragments (all except for C+His)



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
his b1	Default	13/06/2014	11:45	76.46	1.529	0.916	1.67	1.67	50.00	230	0.916	6.965
his b2	Default	13/06/2014	11:45	119.68	2.394	1.279	1.87	1.25	50.00	230	1.920	0.049
his D 95	Default	13/06/2014	11:46	197.30	3.946	2.019	1.95	1.84	50.00	230	2.140	0.016
his x83	Default	13/06/2014	11:47	136.50	2.730	1.432	1.91	1.50	50.00	230	1.819	0.019
his y19	Default	13/06/2014	11:48	171.93	3.439	1.814	1.90	1.50	50.00	230	2.292	0.028
his y20	Default	13/06/2014	11:49	178.39	3.568	1.910	1.87	1.38	50.00	230	2.579	0.049
his z34	Default	13/06/2014	11:49	120.84	2.417	1.312	1.84	1.24	50.00	230	1.945	0.018
his z35	Default	13/06/2014	11:50	115.49	2.310	1.197	1.93	1.16	50.00	230	1.991	0.045

Gelelectrophoresis of colony PCR

-in 1% agarose gel at 120 V

14.06.2014

Glycerinstocks

GS 80-97, 92-95

Miniprep

107: 160 ng/µL

108: 31 ng/µL
109: 110,4 ng/µL
110: 65,2 ng/µL
111: 304,6 ng/µL
112: 231 ng/µL

Restriction for control

digested by XbaI/PstI (1µg DNA or 20µL each sample)

18.06.2014

Restriction

PL93 (mmoX+His)	32 µL (15 mg)
Cut-Smart Buffer	4 µL
Xba I	1 µL
Pst I	1 µL
H_2O	2 µL
∑	40 µL

18.06.2014

Gel- Elution of mmoD + His and mmoZ + His

20.06.2014

Ligation into 6.3.11 LacProm [R0011]+RBS and transformation

23.06.2014

Colony PCR of Lac-RBS- mmoD-His. 24 colonies were picked.

	25x
Template	6,25 µL
5 x GoTaq-Puffer	50 µL
10 mM dNTPs	5 µL
Primer 1	12,5 µL
Primer 2	12,5 µL
GoTaq Polymerase	1,25 µL
MgCl	20 µL
dH_2O	142,5 µL
∑	250 µL

Lac-mmoZ-His was not successful .

Gelelectrophoresis

-gelelctrophoresis in 1,5% agarose gel at 140 V

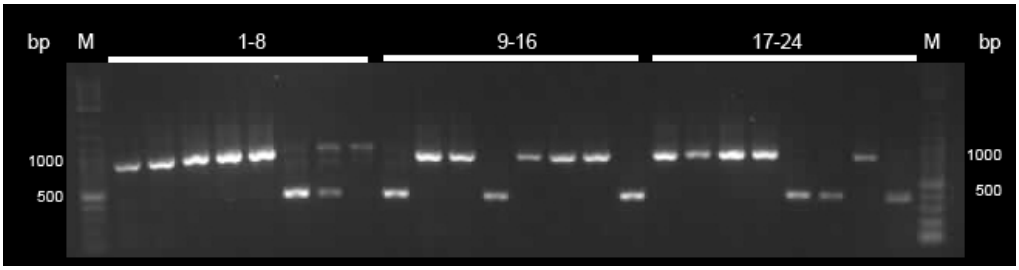


Fig. 2.9 Colony-PCR – 1-24: Lac-RBS-mmoD-HIS (Exp. lenght: ~900)

→ clones 1-5, 8, 10-11, 13-15, 17-20 and 23 are positive

25.06.2014

PCR for new his-tag of mmoX, mmoD and mmoZ

- mmoX (PL 99), mmoD (PL 57) and mmoZ (PL 97) were diluted to a concentration of 10 ng/µL

Restriction of PL 1 (vector TT)

PL 1	60 µL
Cut-Smart Buffer	10 µL
EcoR I	1 µL
Xba I	1 µL
H_2O	28 µL
∑	100 µL

Gelelectrophoresis in 1% agarose gel at 140 V

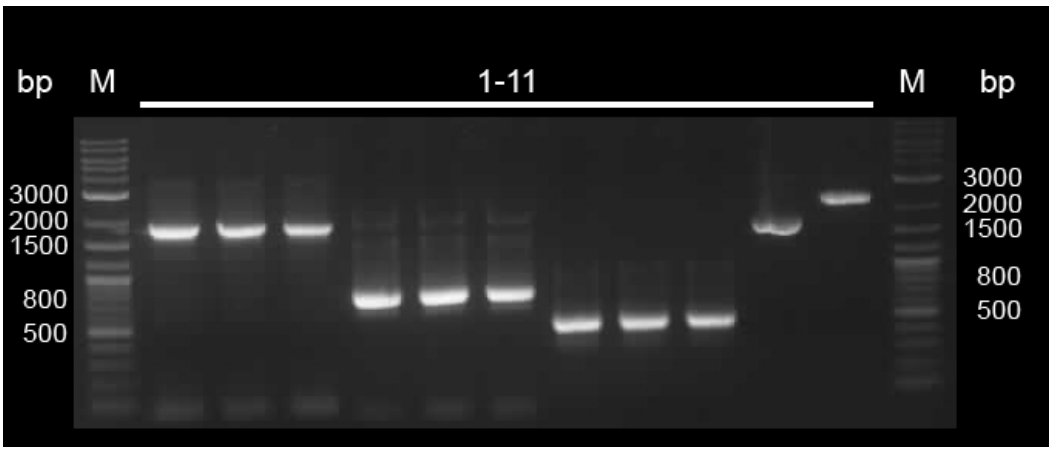


Fig. 2.10 Gel-purification – 1-3: mmoX-HIS (Exp. lenght: 1812); 4-6: mmoD-HIS (Exp. lenght: 739); 7-9: mmoZ-HIS (Exp. lenght: 472); 10: Uncut vector B0015[TT]; 11: EcoRI+XbaI cut B0015[TT]

→ all restrictions are positive

Gelelution

Restriction

Niels, Lukas, Rüdiger

26.06.2014

Ligation of Vector TT + mmoX, mmoD, mmoZ and GUS Reporter

- 4 samples of each

Vector TT (PL1)	1 µL
Insert	7,5 µL
T4 DNA Ligase	0,5 µL
T4 Ligase Buffer 10x	1 µL
∑	10 µL

Gelectrophoresis

27.06.2014

Oliver, Rüdiger

Primer dilution 88/89 1:10

Colony-PCR

	Comment	**Volume per sample [µL]**	**Volume for X samples [µL]**
Template	Colony	0,25	37,5
5 x GoTaq-Puffer	contains NO Mg^{2+}	2	300
10 mM dNTPs	-	0,2	30
Primer 1	10 pmol μL^{-1}	0,5	75
Primer 2	10 pmol μL^{-1}	0,5	75
GoTaq Polymerase	5 U μL^{-1} (contains loading buffer)	0,05	7,5
MgCl	-	0,8	120
dH_2O	-	5,7	855
∑	-	10	1500

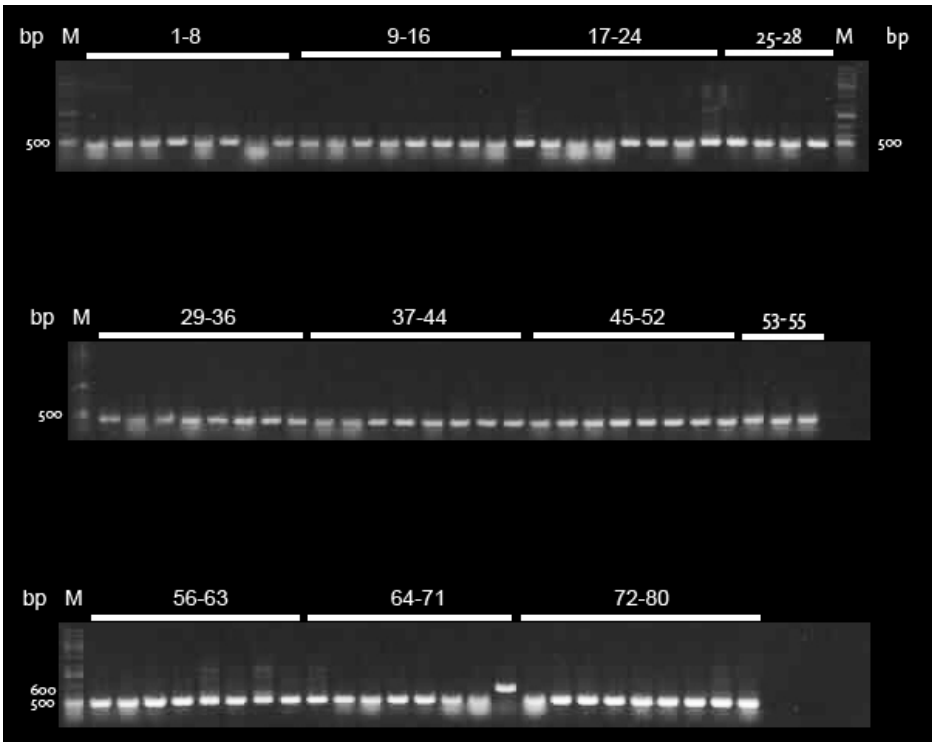


Fig. 2.11 *Colony-PCR – **1-36**: mmoZ (Exp. lenght: 1020); **37-73**: GUS (Exp. lenght:2064 ; **74-80**: mmoD (Exp. lenght: 761)*

→ only clone 81 of mmoD is positive

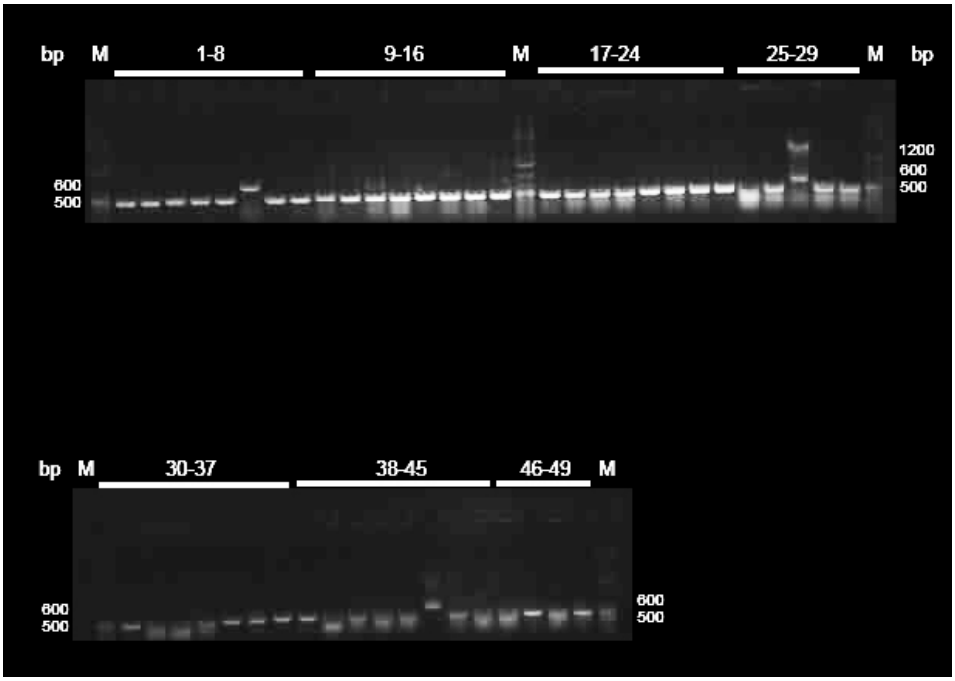


Fig. 2.12 *Colony-PCR – **1-16**: mmoD (Exp. lenght: 1020); **17-49**: mmoX (Exp. lenght:2064)*

→ clone 6 of mmoD and clone 11 and maybe 26 of mmoX are positive

mmoZ/GUS no positive clones

Plasmid 97 Trafo was again amplified and eluted for new transformation

GUS cut again and transformation

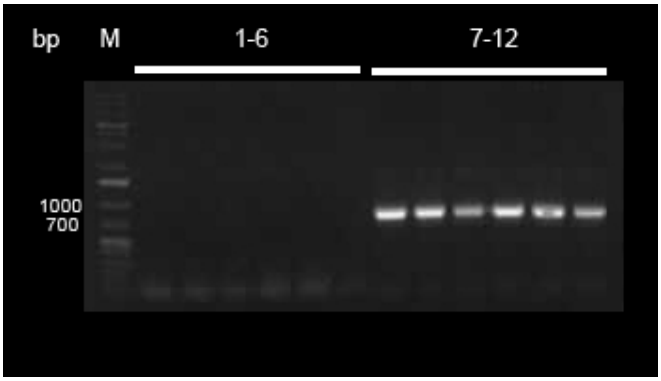


Fig. 2.13

iGEM2014-8 PCR mmoZ 739 bp(Plasmid 97)mmoX 1812 (Plasmid 99 negativ) His

28.6.14

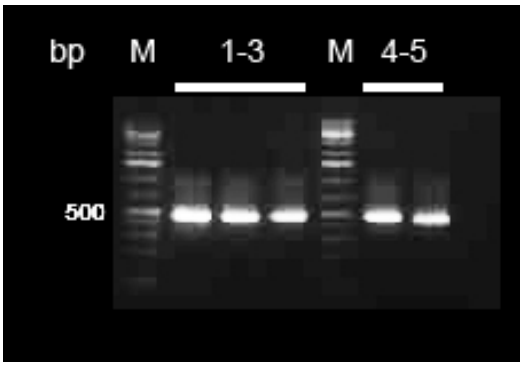


***Fig. 2.14** Colony-PCR – **1-3**: mmoD-HIS-TT; **4-5**: mmoX-HIS-TT*

mmoD Fragment für HIS+TT (bande 2-4) und Bande und 7 YRC Glycerin mmoX 4849 bande 9 und 10 neg

30.06.2014

Niels, Lukas

Test restriction

Restriction for testing the integrity of plasmids and inserts

Plasmid 99 and 49	10 µL
Cut-Smart Buffer	10 µL
Res 1 SpeI	0,5 µL
Res 2 PstI	0,5 µL
H_2O	79 µL
∑	100 µL

Gelectrophoresis

-gelelctrophoresis in 1% agarose gel at 140 V

Fragment	PL 49 uncut	PL 49 cut		PL 99 uncut	PL 99 cut
Expected length [bp]					
Band-No.	1	2	3	4	5
Positive			0		

Restriction

mmoD , mmoZ and Gus	10 µL
Cut-Smart Buffer	10 µL
Res 1 EcoRI-HF	0,5 µL
Res 2 SpeI	0,5 µL
H_2O	19 µL
∑	40 µL

PCR

Gene	**Plasmid**	**Primer**	
		forward	**reverse**
mmoX	99	88	93

	Comment	**Volume per sample [µL]**
Template	Plasmid 99	0,8
Pfu-Buffer	contains Mg^{2+}	5
10 mM dNTPs		1
Primer 88	10 pmol $µL^{-1}$	1,25
Primer 93	10 pmol $µL^{-1}$	1,25
Pfu-Polymerase	5 U $µL^{-1}$ (contains loading buffer)	1
dH_2O		39,7
∑		50

Colony-PCR

	Comment	**Volume per sample [µL]**	**Volume for 4 samples [µL]**
Template	mmoD(7/22) mmoX(11)	0,25	1
5 x GoTaq-Puffer	contains NO Mg^{2+}	2	8
10 mM dNTPs		0,2	0,8
Primer 1	10 pmol μL^{-1}	0,5	2
Primer 2	10 pmol μL^{-1}	0,5	2
GoTaq Polymerase	5 U μL^{-1} (contains loading buffer)	0,2	0,8
MgCl		0,8	3,2
dH_2O		5,7	22,8
∑		10	40

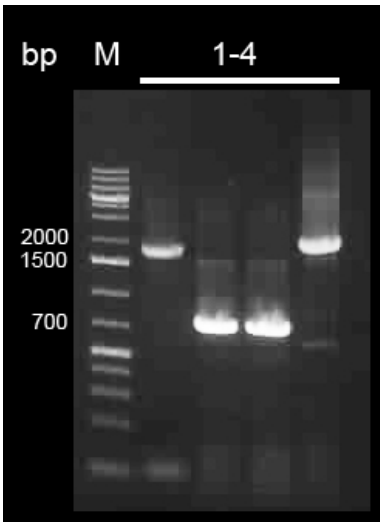


Fig. 2.15 *Colony-PCR – **1**: Lac-RBS-mmoX (Exp. lenght :1800); **2**: mmoD colony 7 (Exp. lenght:750); **3**: mmoD colony 22 (Exp. lenght:750); **4**: mmoX colony 22(Exp. lenght:2000)*

01.07.2014

Oli

Plasmid-mini-prep

➔ of mmoD clone 7 and 22, and mmoX clone 11

Colonie PCR

-of mmoD-His and mmoZ-His (30 colonies each were picked)

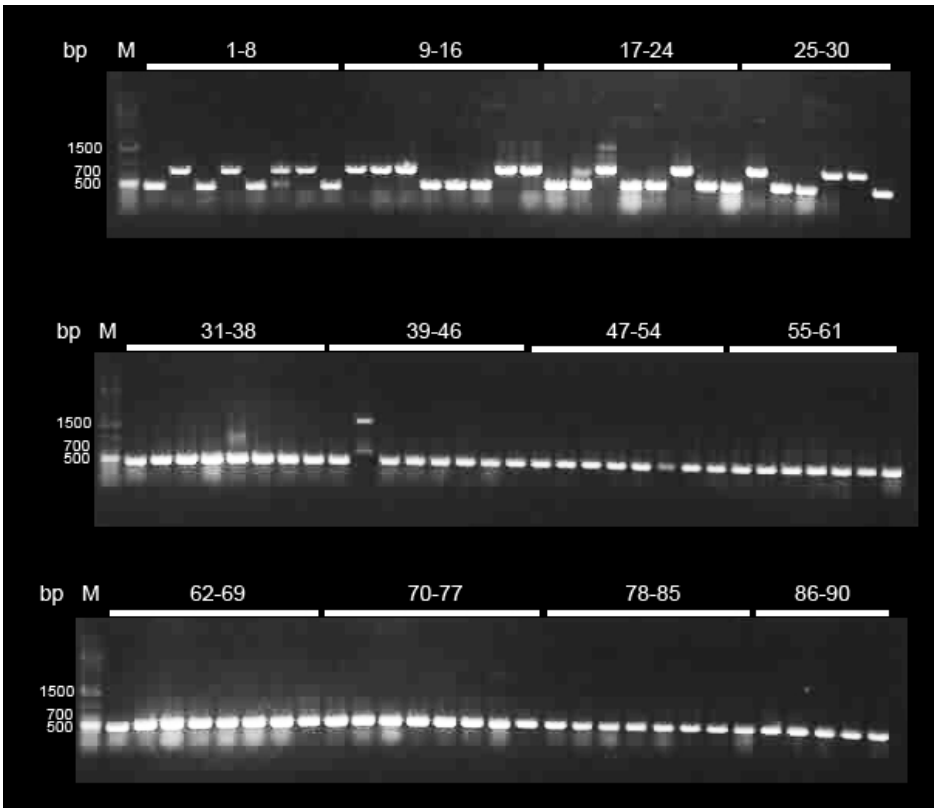


Fig. 2.16 *Colony-PCR – **1-30**: mmoD-HIS; **31-60**: mmoZ-HIS; **61**: GUS colony 3; **62**: GUS colony 1; **63**: GUS colony 2; **64-90**: GUS colonies 4-30*

Overnight culture

➔ of mmoD-HIS 2,4,7 and mmoZ-HIS 5

02.07.2014

Oli, Rüdiger

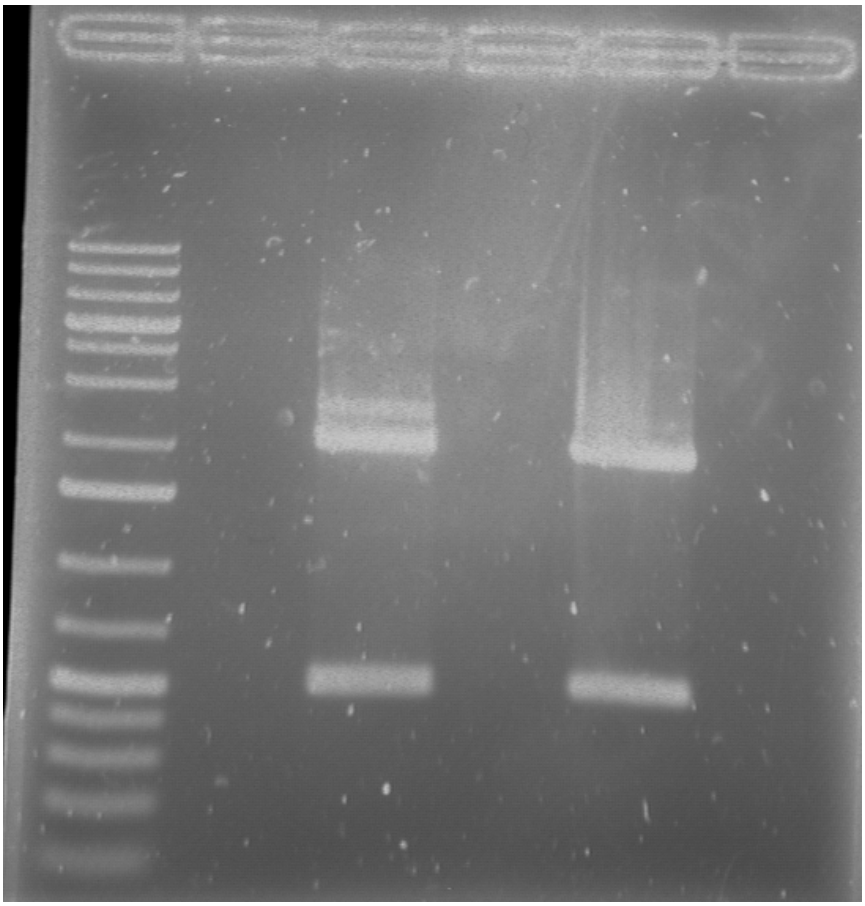
Glycerin stocks and plasmid preparation of over night cultures.

Restriction of Lac-mmoD -His clone 7 and 22 with XbaI and PstI.

03.07.2014

Oli

Purification of restriction via 1,5% agarose gel at 140 V.



Fragment	M	-	D7	-	D22
Expected length [bp]	1kb GeneRuler plus		458		458
Band-No.	1	2	3	4	5
Clone-No.	1	2	3	4	5
Positive	+		+		+

Ligation

mmoD-His-TT (cut with X + P)	µL 14
Vector 6.3.11 (S + P)	µL 3
T4 DNA Ligase	µL 1
T4 Ligase Buffer	µL 2
∑	µL 20

For 1 hour at RT.

Transformation and platting.

04.07.2014

Oli, Zen-Zen

Sequenzing of mmoZ-His final failed. Therefore, some more clones of the master plate are picked, to check for positive clones.

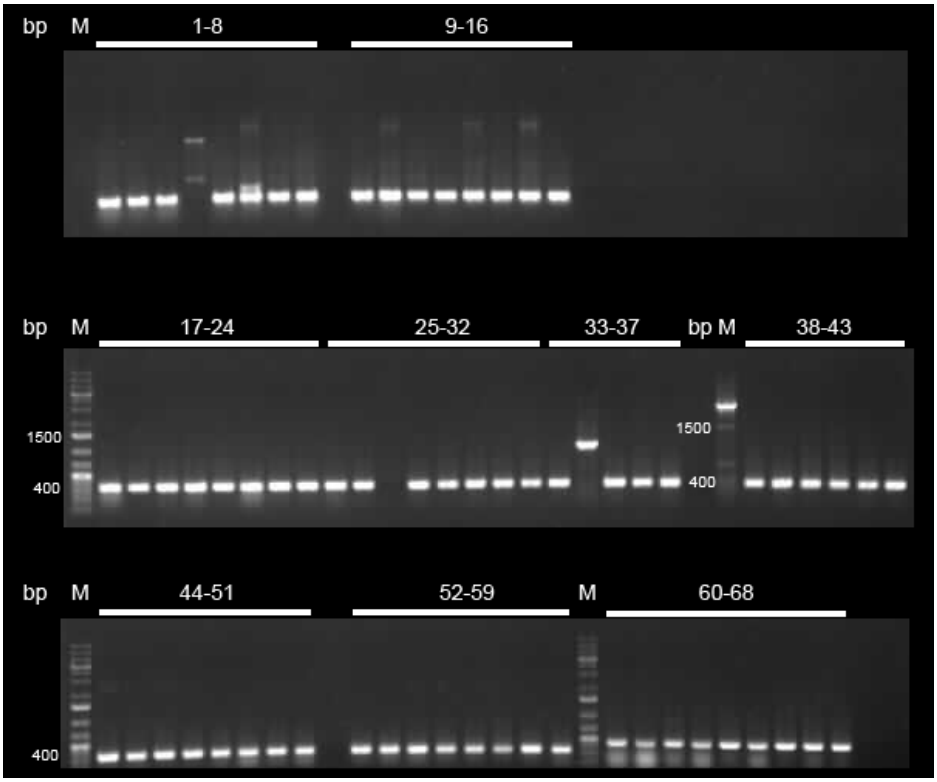


Fig. 2.17 *Colony-PCR – final Lac-RBS-mmoZ-HIS*

➔ no positive clones…

Colony PCR of the final mmoD-His construct

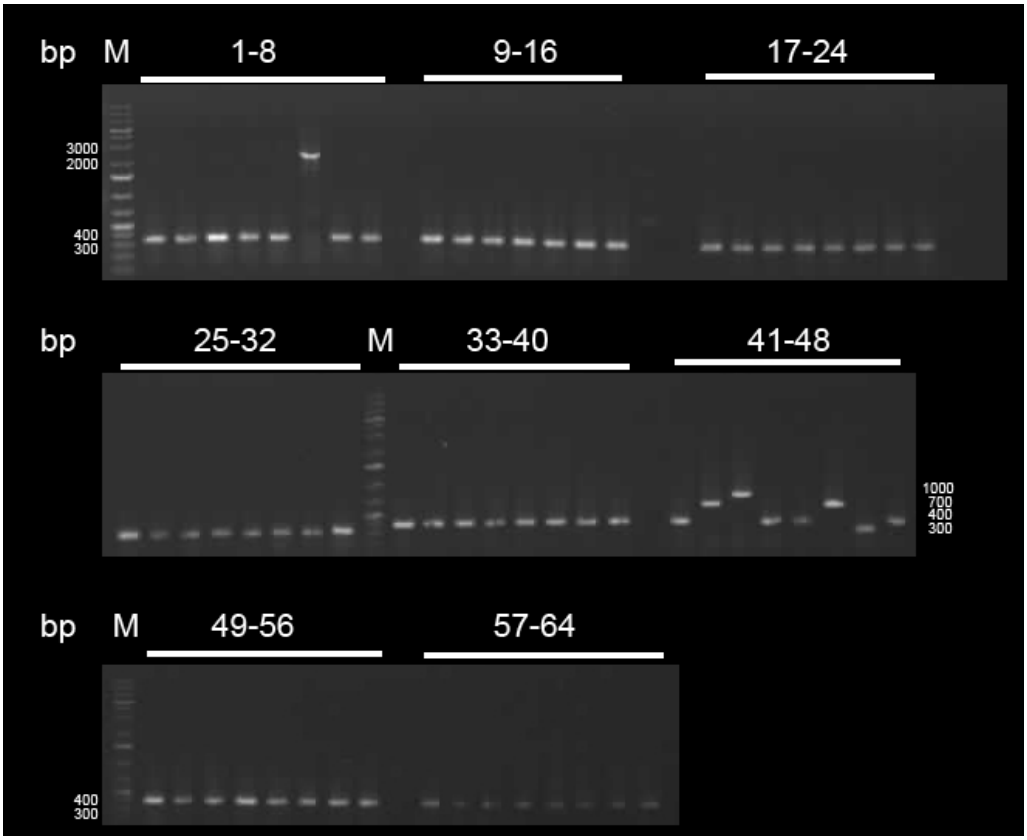


Fig. 2.18 Colony-PCR – Lac-RBS-mmoD-HIS

➔ clone 42, 43 and 46 are picked for over night cultures.

05.07.2014

Carsten, Oli, Steffen

Over night cultures were not successful, because the incubator was switched off by unknown…

Cultures are inoculated again.

06.07.2014

Carsten, Oli, Christian

Plasmid preparation of over night cultures.

DNA concentration is much too low, therefore the over night cultures are inoculated again. Same clones and GS as the day before. In addition to that over night cultures of the following GS will be inoculated:

Lac-RBS-vector (GS: 35)

mmoZ-His-TT (GS:63, Plasmid 65)

Colony-PCR of all remaining colonies of the Lac-RBS-mmoZ-His-TT-masterplate from 01.07.2014: Clone 1,2,10, 13-30.

07.07.2014

Oli, Carsten

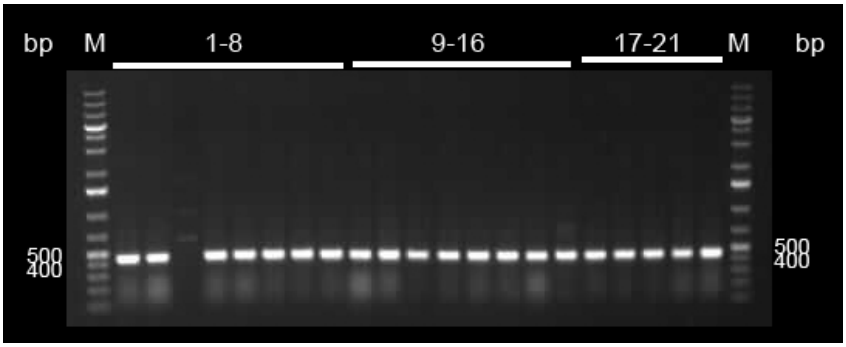


Fig. 2.19 *Colony-PCR – 1-21: final Lac-RBS-mmoZ-HIS (Exp. lenght: ~1000)*

➔ all clones are negative

In order to finally find a positive clone, 60 colonies are picked for colony PCR from agar plate “mmoZ plasmid 97 in TT vector”, with primer 88 und 89.

1,0% agarose gel electrophoresis at 140V:

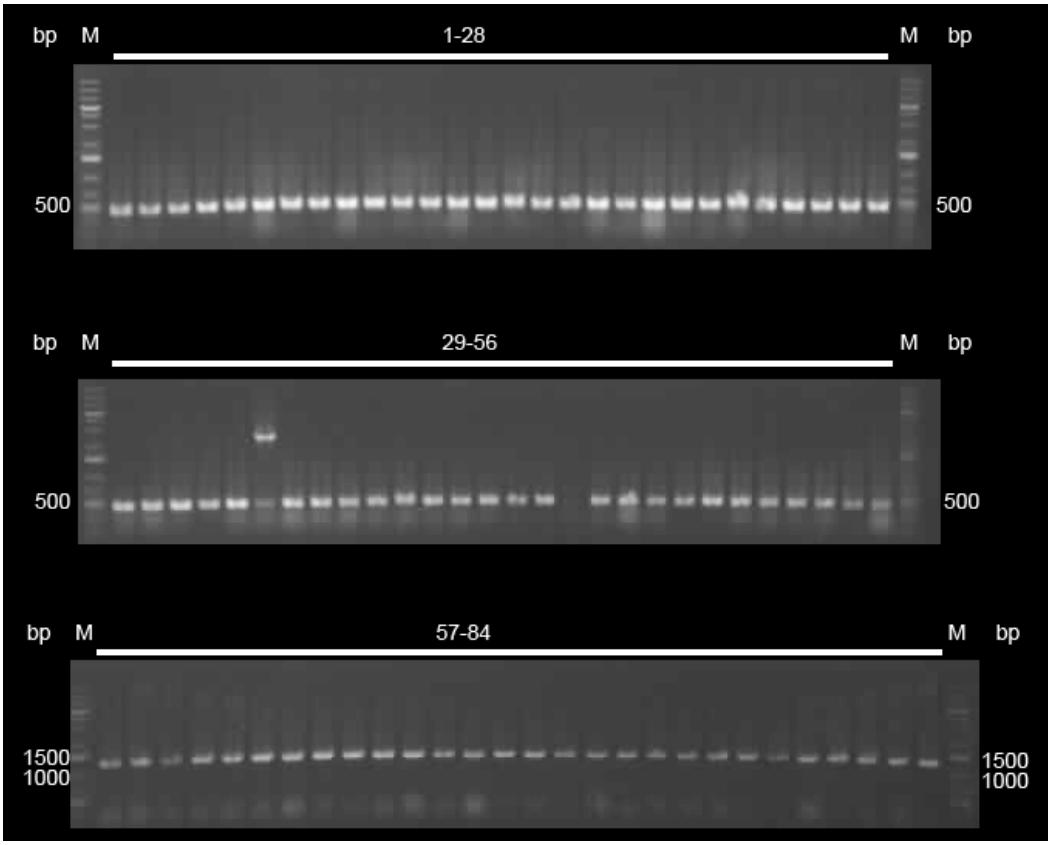


Fig. 2.20 Colony-PCR – 1-21: final Lac-RBS-mmoZ-HIS (Exp. lenght: ~1000)

➔ No positive clones… the restriction and ligation of plasmid 97 into the TT-vector will be repeated tomorrow.

Plasmid preparation of the over night cultures was successful. DNA of mmoD-His finale construct of clone 10, 11 and 14 are send for sequencing

08.07.2014

Oli, Carsten

Because there were no positive clones on the current transformation plate, the last step of the construction of the mmoZ-His final construct is repeated.

Restriction

Fragment: lac-RBS-mmoZ-His	µL 43 (45,5ng/ µL)
Cut-Smart Buffer	5 µL
EcoRI	1 µL
SpeI	1 µL
H_2O	0 µL
∑	**50 µL**

Restriction for 3h at 37°C

Gelpurification of lac-RBS-mmoZ-HIS

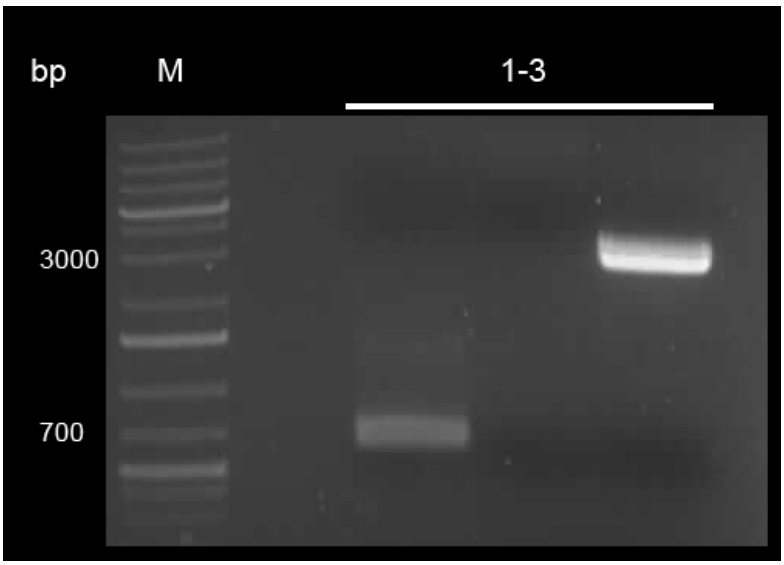


Fig. 8.21 *Gel-purification – **1**: Lac-RBS-mmoZ-HIS; **2**: Backbone; **3**: RFP2*

➔ The LAC-RBS-mmoZ-HIS band is cut out and purificated with promega wizard column kit
- Concentration: 9 ng/µl

Ligation

➔ 4 µl of cut TT-Vector , 14 µl LAC-RBS-mmoZ-HIS (9 ng/µl), 2 µl T4 Ligase and 2 µl T4 buffer and incubated at 16 °C over night

09.08.2014

Oli

Transformation of over night ligation of Lac-RBS-mmoZ-His-TT.

Sequencing results of mmoD-His finale construct of clone 10, 11 and 14: all three sequences are wrong. No positive clone.

10.07.2014

Oli

The transformation plate is showing many colonies.

In order to find a positive clone a colony-PCR of 64 colonies is performed with primer 83 and 84 (binding on prefix and suffix region).

1% agarose gel electrophoresis at 140V:
*Gel-purification – **1**: Lac-RBS-mmoZ-HIS; **2**: Backbone; **3**: RFP2*

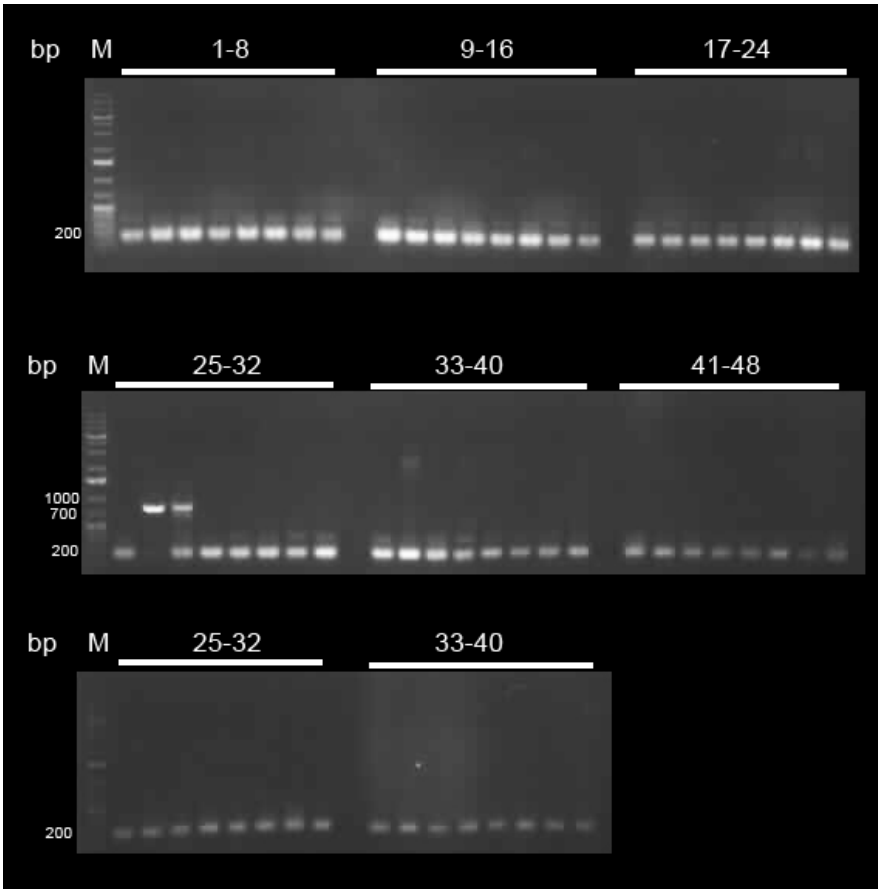


***Fig. 2.22** Colony-PCR – **1-40**: Lac-RBS-mmoZ-HIS-TT*

→ Clone 26 and probably clone 27 are positive.

Over night cultures of both positive clones are prepared.

Restriction of mmoD-His-TT (clone 22) with XbaI and PstI, used DNA: 10 µg.

→ Gelpurification

11.07.2014

Oli, Carsten

Plasmid preparation of over night cultures (clone 26 and 27 of Lac-RBS-mmoD –His-TT)

DNA concentrations: 26: 451,61 ng/µL; 27:591,57 ng/µL

→ both are send for sequencing.

Purification from gel of mmoD-His-TT and ligation into Lac-RBS-vector.

12.07.2014

Maren, Carsten

Check transformation plate: Enough colonies grown on 100 µl- and rest-plate
32 colonies are picked for colony-PCR.

➔ Error: Colony-PCR-Program was altered to an annealing temperature auf 38 °C (PCR maybe not successful)

<u>Gelelectrophoresis of Colony-PCR</u>

-In 1% agarose gel at 140 V for 40 minutes

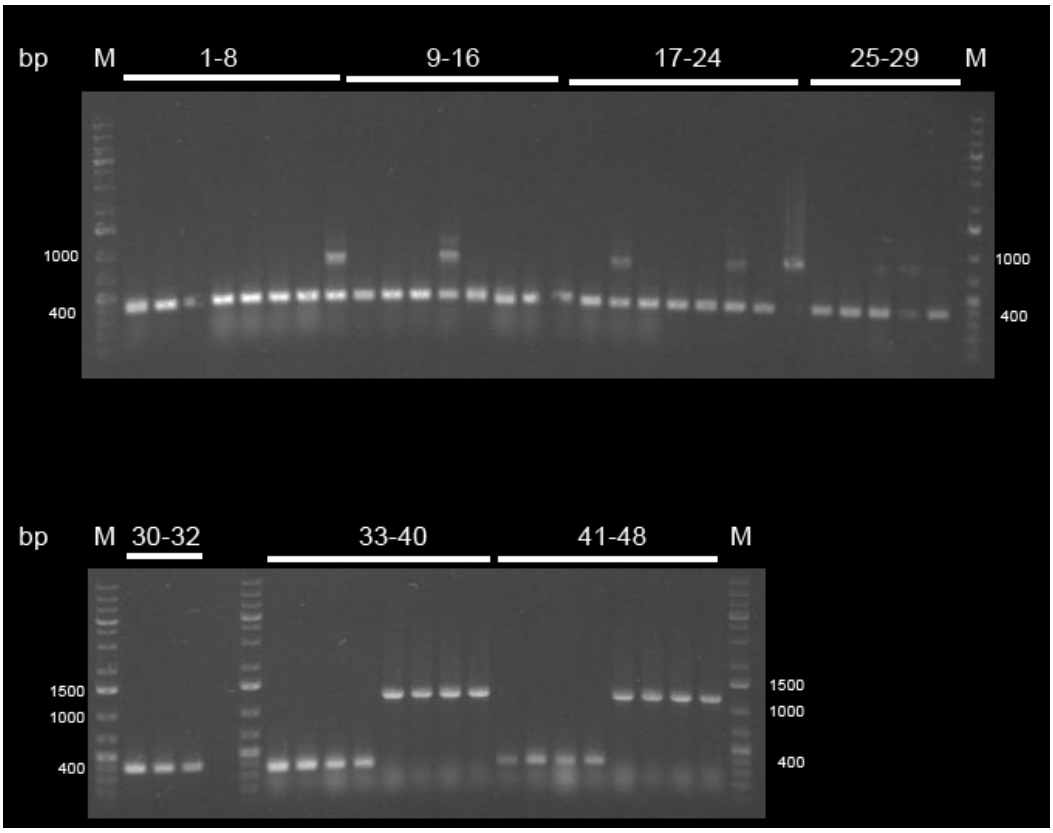


*Fig. 2.23 Colony-PCR – **1-48**: Lac-RBS-mmoD-HIS-TT (Exp. lenght: ~450)*

→Positive Clones:8,12,18,22,24,28.

→Clones used for plasmid prep: 24 and 28

13.07.2014

Melanie

<u>Overnight cultures</u>

➔ Of Clones 24+25 in 5ml 2YT-medium plus Chloramphenicol

14.07.2014

Oli, Anna, Carsten

Plasmid preparation of over night cultures. Unfortunately the DNA concentration of the preparation was too low, therefore the over night cultures are inoculated again.

15.07.2014

Nils, Oli

Plasmid preparation of over night cultures.

Restriction with NotI for 3h to test the fragments for correct size

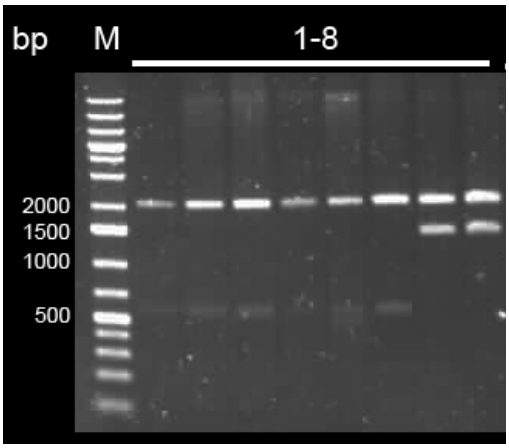


Fig. 2.24 *Lac-RBS-mmoD-TT (Exp. length 572)*

→ clones 1-4 and 6 are positive

Lac -RBS-mmoD -His-TT clone 8, 12, 18 and 28 are correct

➔ mmoD-His final Clones 8 and 12, as well as mmoZ-His-Final clone 26 (see 11.07.2014) are send for sequencing

Overnight culture of two (8 and 12) correct clones of each construct 3x5 ml 2xYT-Ca G

17.07.2014

Oli

Results of sequencing:

The final mmoD-His constructs of clone 8 and 12 show no useful data. It is very likely that both are double clones, including one empty vector. Therefore the sequencing result could not be used for analysis.

The final mmoZ-His construct (which was sequenced with re rev. primer this time) still shows the insertion of an A at the beginning of the His-Tag, causing a frameshift. We are going delete the A by mutation.

18.7.2014

Anna, Melanie, Christian

To identify Lac-mmoD-His –TT positiv clones – colony PCR was performed (Primer 88/89)

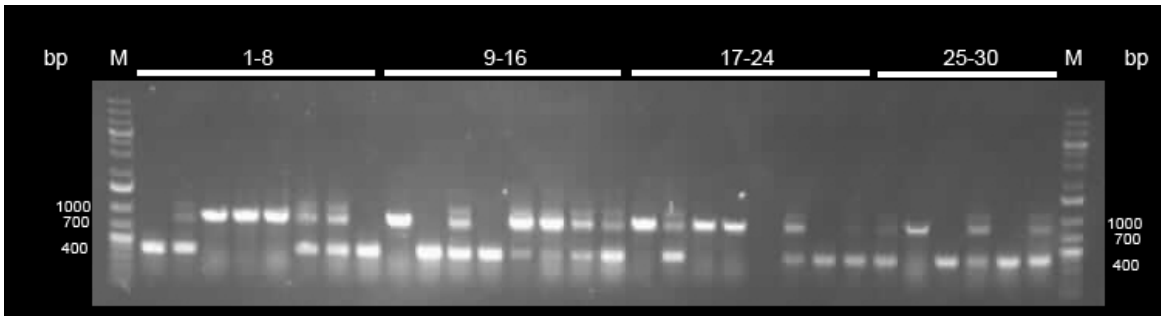


*Fig. 2.25 Colony-PCR – **1-30**: Lac-RBS-mmoD-HIS-TT*

→ clones 3-5 and 19-20 are positive

Over night cultures (5mL) are inoculated with the positive clones 3, 4, 5, 19 and 20.

19.07.2014

Christian, Oli

Plasmid preparation of over night cultures and preparation of glycerin stocks of all five positive clones from yesterday.

21.07.2014

Oli

All five positive clones are sent for sequencing.

23.04.2014

Overnight culture of mmoD-His clone 4 (sequence OK) was inoculated

24.07.2014

Melanie, Christian, Oli

Plasmid prep and Glycerolstock of mmoD –His was done.

➔ His construct of mmoD is complete. The only His-construct missing is mmoZ.

Mutation PCR of mmoZ-his final clone 26 (plasmid 177) with primer 123 and 124:

Reaction buffer Phu 5x	10
dsDNA template 50 ng	1 (dilution 1:9)
Primer 1	1,25
Primer 2	1,25
dNTP mix	1
ddH_2O	34,5
Phusion Polymerase	1
∑	**50**
Used Primers	123/124

Restriction of PCR sample with DPNI, to digest templateDNA over night.

25.07.2014

Oli

Inactivation of DPNI restriction of mutation PCR of the final mmoZ-His construct via heat shock (80°C for 20 min). Afterwards transformation into competent E. coli cells.

28.07.2014

Steffen

Inoculation of over night cultures of 4 clones mmoZ-His(10 mL).

29.07.2014

Oli

Glycerin stock of all four clones and plasmid preparation of all over night cultures.

All four samples are send for sequencing (VR primer 89) in order to check whether the mutation PCR was successful.

31.07.2014

Niels, Melanie, Oli

Check of sequencing results of final mmoZ-His constructs (after mutation PCR):

Clone 3 is positive! The colony PCR was successful. All other clones are discarded.

All six His-constructs are complete!

gene	GS	Plasmid No.
mmoB	84/85	114/115
mmoC	92/94	107/109
mmoD	141	180
mmoX	118	142
mmoY	83	113
mmoZ	151	192

27.08.2014

Oli

In order to cotransform our His constructs with the chaperons, the His constructs need to contain a different resistance. We will clone the His constructs into a backbone with AmpR.

The Lac-mmo-His-TT constructs are digested with EcoRI HF and PstI.

Fragment mmo-His (of all mmo genes but X)	26 µL (~4-5 µg)
Cut-Smart Buffer	3 µL
EcoRI-HF	1 µL
∑	**30 µL (incubation for 1h at 37°C)**
+ H_2O	5 µL
+ PstI	1 µL
+ Puffer 3.1	4 µL
∑	**40 µL (incubation for 1h at 37°C)**

For mmoX:

Fragment mmoX-His	50 µL (~4 µg)
Cut-Smart Buffer	6 µL
EcoRI-HF	1 µL
H_2O	3 µL
∑	**60 µL (incubation for 1h at 37°C)**
+ H_2O	2 µL
+ PstI	1 µL
+ Puffer 3.1	7 µL
∑	**70 µL (incubation for 1h at 37°C)**

-Gelelectrophoresis in 2% agarose gel for mmoX and 1% agarose gel for purification of digested fragments

Fig. 16.1. *Gel-purification – **1-2**: Lac-mmoB-HIS-TT (Exp. Length 662 bp); **3-4**: Lac-mmoD-HIS-TT (Exp. Length 548 bp); **5-6**: Lac-mmoC-HIS-TT (Exp. Length 1283 bp); **7-8**: Lac-mmoY-HIS-TT (Exp. Length 1406 bp); **9-10**: Lac-mmoZ-HIS-TT (Exp. Length 749 bp) – all cut with EcoR1 and Pst1*

Fig. 16.2 *Gel-purification –* ***1-5****: Lac-mmoX-HIS-TT (Exp. Length 1820 bp) cut with EcoR1 and Pst1*

→ lower bands are cut out and stored at -20 °C

Fig. 16.3 *Gel-purification –* ***1-5****: Lac-mmoX-HIS-TT (Exp. Length 1820 bp) cut with EcoR1 and Pst1*

Christian, Rüdiger

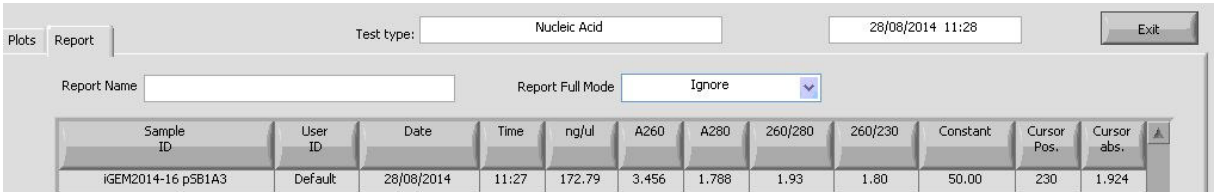
28.08.2014

<u>Gelelution</u>

Of the His-constructs that were cut out of agarose gel on 27.08.2014 using Promega PCR Wizard.

<u>Plasmid Prep</u>

Of pSB1A3 from GS 170



Restriction

Of pSB1A3 with EcoRI and PstI

pSB1A3	77 µL (~13 µg)
Cut-Smart Buffer	10 µL
EcoRI-HF	2 µL
H_2O	11 µL
∑	**100 µL (incubation for 1h at 37°C)**
+ H_2O	6 µL
+ PstI	2 µL
+ Puffer 3.1	12 µL
∑	**120 µL (incubation for 1h at 37°C)**

-Gelelectrophoresis for purification of the backbone

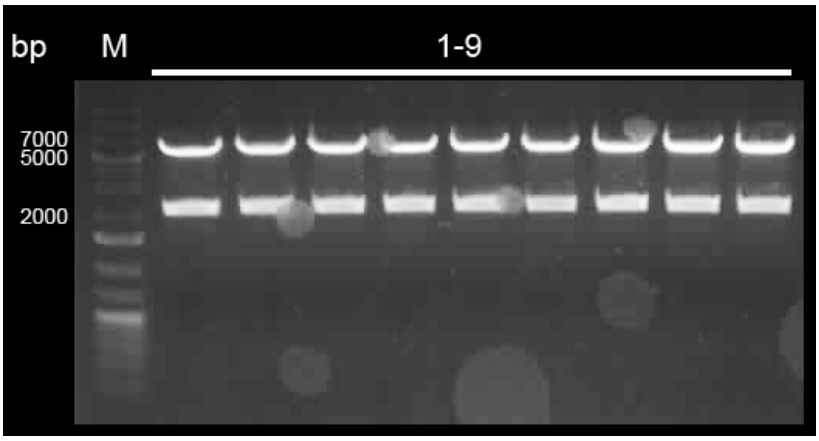


Fig. 16.3 *Gel-purification –* ***1-9****: Vector pSB1A3 cut with EcoR1-HF and Pst1 (Backbone is the lower band)*

→ the backbone (lower bands) were cut out and eluted with Promega Wizard (77 ng/µL)

Ligation

Of the His-constructs in pSB1A3

His-construct	7 µL
psB1A3	2 µL
T4 DNA Ligase	0,5 µL
T4 Ligase Buffer	1 µL
∑	10,5 µL

→ ligation over night at RT

Rüdiger

29.08.2014

Inactivation

of ligation at 65 °C for 10 min.

transformation

01.09.2014

Melanie, Anna, Oli

Colony PCR of all transformed his constructs.

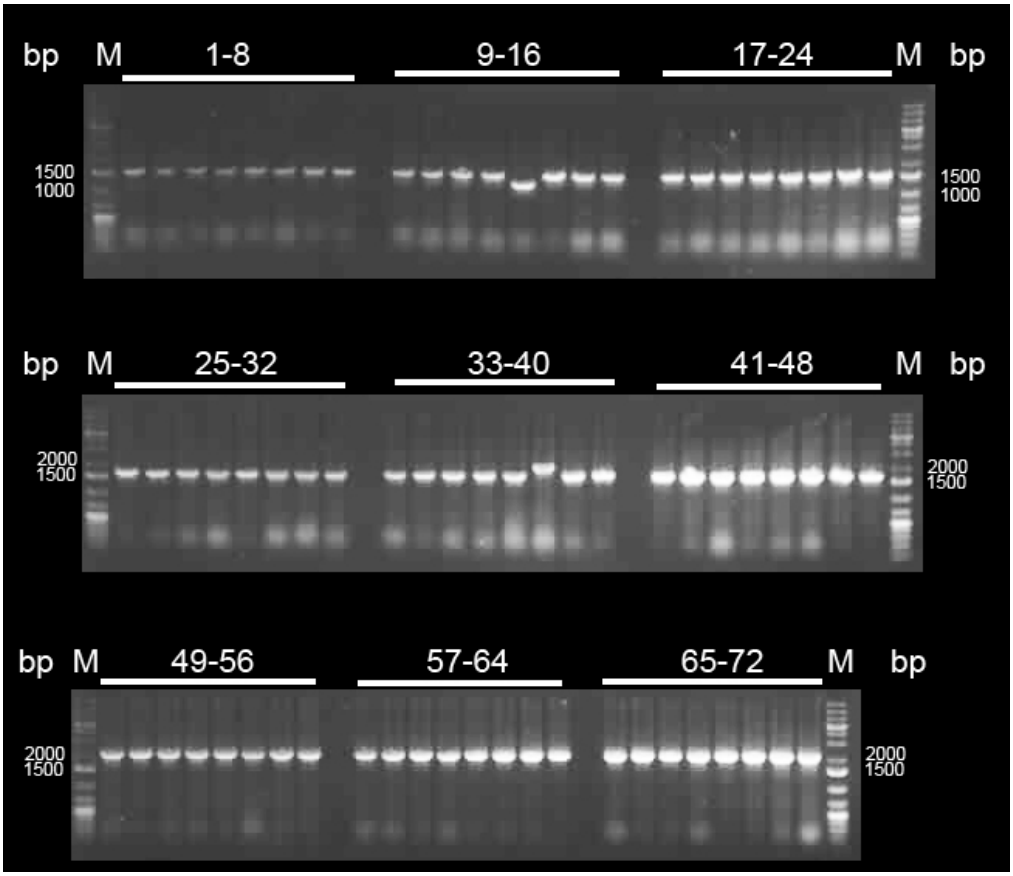


Fig. 16.5 *Colony-PCR – **1-24**: Lac-mmoC-HIS-TT in pSB1A3; **25-48**: Lac-mmoY-HIS-TT in pSB1A3; **49-72**: Lac-mmoX-HIS-TT in pSB1A3*

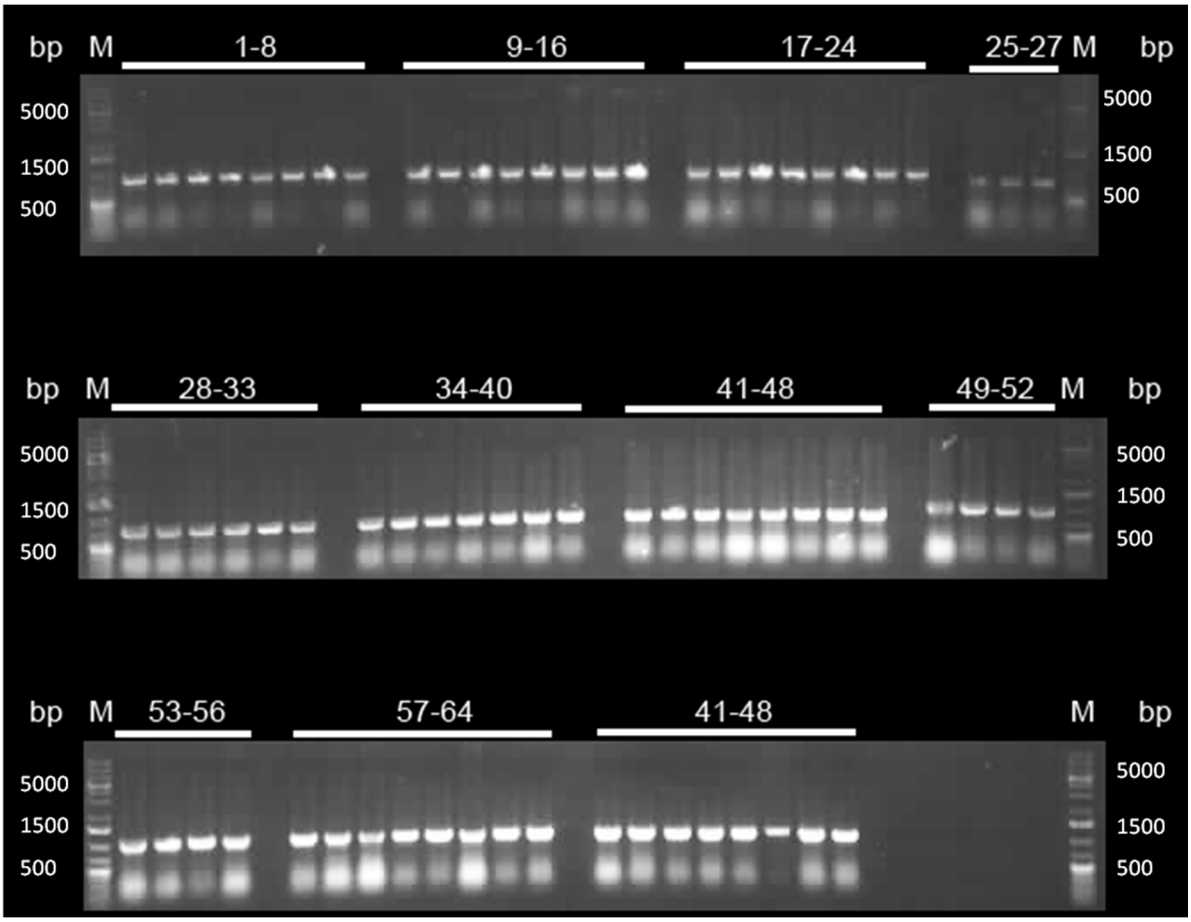


Fig. 16.4 *Colony-PCR –* ***1-24****: Lac-mmoC-HIS-TT in pSB1A3;* ***25-48****: Lac-mmoY-HIS-TT in pSB1A3;* ***49-72****: Lac-mmoX-HIS-TT in pSB1A3*

→ positive clones for all six his constructs in pSB1A3 vector.